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(54) DETERMINATION OF THE α-AMYLASE ACTIVITY OF A MATERIAL

(71) We, F. HOFFMAN-LA ROCHE & Co., AKTIENGESELLSCHAFT, a Swiss Company of 124-184 Grenzacherstrasse, Basle, Switzerland, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to a method of determining the α-amylase activity of a material, and to a reagent test kit for such a determination.

In many diseases wherein substantially identical symptoms appear in the patient, differential diagnosis is difficult. For this reason, reliable chemical diagnostic tests are sought and used with increasing frequency. For example, one such pair of clinical diagnostic problems is a differentiation between appendicitis and acute pancreatitis. In efforts to improve the accuracy of determining which disease is present, a reliable chemical method is needed to accurately determine either the serum or urine amylase activity. This activity, which in humans is mostly the product of exocrine pancreatic function, is significantly increased in acute pancreatitis.

Amylase is a group of enzymes comprising α-amylase and β-amylase. These enzymes cause the hydrolysis of starch, therefore, chemical methods of determining the by-products of the hydrolysis are feasible. The hydrolysis of amylose, the linear α-1,4-glucosidic starch component, can be used to measure α- and β-amylase activity. The products of the enzymatic hydrolysis of amylose by these amylases are maltose and glucose or mixtures thereof. In the case of α-amylase, the main product is a mixture of maltose and glucose, while in the case of β-amylase, the main product is maltose. Thus, tests have been developed which measure the activity of the enzyme in terms of the amount of maltose and/or glucose produced. These tests are difficult to carry out and are unreliable since reproducible results are generally not attainable.

This invention relates to methods for determining α-amylase activity and a reagent test kit for such a determination. More particularly, this invention relates to the determination of α-amylase activity by a color test utilizing as the substrate, a water-insoluble complex of a water-soluble dye and an amylose-containing material. As used hereinafter "amylase" refers to both α- and β-amylase.

It has been discovered that α-amplase activity can be measured with excellent reproducibility, e.g., within 2-4% mean deviation, by measuring the color intensity produced when a water-soluble dye, insolubilized by combination with amylose, is solubilized in an aqueous medium by the action of amylase. The color of the aqueous medium is measured and compared with a control or standard to determine the degree of solubilization, which is a measure of the hydrolysis, effected by the amylase and thus a measure of its activity.

The dyes which are suitable for use in this invention for complexing with amylose are those which are water-soluble and are reactive with cellulose, e.g., the so-called "reactive dyes". The preferred types are the reactive monochlorotriazine dyes, especially suitable are those which contain an anthraquinone disulfonate moiety. The dyes of this type are known to the art and can be illustrated by the following: Cibacron (Registered Trade Mark) Brilliant Orange G.E. (Reactive Orange 5) and Cibacron (Registered Trade Mark) Brilliant Blue F3GA (Reactive Blue 2).

The "reactive dyes" are characterized as being water-soluble, as containing a reactive halogen substituent, e.g., they are de-

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rived from cyanuric chloride in which two of the available chlorine atoms are replaced, and as containing an anthraquinone disulfonate moiety. The dyes are usually available as their heavy metal salts, e.g., copper, manganese and the like.

The insoluble complex suitable for use in this invention is prepared by reacting the reactive dye with amylose or an amylose-containing material, e.g., a starch. Typical amylose-containing materials are starches of tuber, cereal or grain origin, e.g., corn, potatoes, wheat or rice.

The dye and amylose-containing material can be reacted together in alkaline aqueous medium in any convenient proportions. The reactive amounts of the reactants are not critical since the reactants combine in a constant manner and any excess reagent present can be removed. Thus, changes in the relative amounts of reactants will not change the character of the reaction product. Accordingly, it is preferred that an excess of one of the reactants be present to insure as complete a reaction as possible.

The complex can be formed by reacting the dye and amylose-containing material in a weakly alkaline aqueous medium at elevated temperatures, e.g., over 50°C, preferably from 50°C to 90°C. If the reaction conditions are too severe, e.g., too hot or too alkaline, the halogen atom on the dye will preferentially react with the hydroxyl ion on the basic medium rather than the amylose moiety. In order to achieve a good yield in short reaction times, it is desirable to add first a soluble salt, e.g., sodium sulfate, calcium chloride, sodium chloride, potassium chloride and the like, sodium sulfate being preferred. After addition of the salt, it is desirable to add a base to bring the pH to at least 9-12 in order to initiate and drive the reaction. This can be accomplished with a base such as, e.g., trisodium phosphate. Other bases are suitable, however, trisodium phosphate is preferred since it permits higher yields.

The product is a stable insoluble complex which can be recovered by conventional means, e.g., filtration, centrifugation, dialysis and the like, and is the same color as the dye. The dye portion of the product is attached to a hydroxyl group of a sugar moiety of the amylose, generally, one dye molecule for from about every seven to about every ten sugar moieties as measured by the ratio of the amount of solubilized dye containing fragments to the reducing substances measured as glucose produced by hydrolysis with endo- and exo-amylase enzymes.

The method of determining the amylase activity according to this invention must be carried out within the limits of the conditions set forth in order to be effective, accurate and reproducible.

The enzyme source may be any material which can contain α-amylase activity, e.g., biological fluids or tissues, fungal, bacterial, or plant extracts. Examples of materials which may contain α-amylase activity are blood serum, urine, saliva and the like. For convenience, this invention will be described using biological fluids, e.g., pancreatic extract, to illustrate the assay for α-amylase activity.

The test to determine α-amylase activity is carried out by suspending the dye-starch complex in a substantially neutral aqueous buffer containing an alkali metal chloride catalyst, warming the suspension, adding the test solution and incubating the mixture for a predetermined length of time, quenching the reaction, removing the supernatant and comparing its color with a standard or control to measure the amount of solubilized dye-containing material. The color produced by the solubilized dye-containing material is a manifestation of the enzyme activity.

The amounts of the various ingredients used in the enzyme activity test are important but not critical to the operability of the test. However, it is preferred to use as small an amount of each ingredient as possible in order to have an economically feasible test. The relative proportions of ingredients are, however, critical to the operability of the process as are the pH and temperature conditions. The time is important only insofar as it is convenient, economical and consistently used.

The reaction conditions for determining the enzyme activty can be varied to provide either a static or dynamic system. In the static system, which is the preferred system, small volumes are used during incubation, eliminating the need for agitation. Thus, the preferred reaction conditions for determining α-amylase activity are those which are directed to obtaining from 6 to 10 ml final volume to be used for spectrophotometric measurement. Thus it is preferred to use about 200 mg of insoluble dye-amplose complex suspended in 0.8 ml of 0.04 M phosphate buffer at a pH of 6.9 to 7.1, preferably 7.0, containing 0.05 M/l NaCl at 37°-40°C, and 0.1 to 0.4 ml of enzyme solution, preferably 0.1 ml. If the amount of dye-amylose complex is increased, then the remaining materials must be increased proportionately.

The relative amount and concentration of the phosphate buffer in the static system is operable within somewhat narrow ranges. Thus, for every 200 mg of substrate there can be present, in the α-amylase assay, 0.7 to 1.5 ml of 0.04 M phosphate buffer. The volume and concentration of buffer present however should be sufficient to maintain the

pH from 6.9 to 7.1, preferably 7.0, throughout the course of the assay.

The concentration of sodium chloride present is critical in the test and should not be less than 0.01 M/l, preferably 0.05 M. Other alkali metal chloride catalysts, e.g., potassium chloride, can be used, but sodium chloride is preferred because of its availability and low cost.

In the dynamic system, wherein special equipment suitable for agitating the system is required, volumes larger than those used in the static system are used during incubation. The volume increase is effected by increasing the volume of buffer. Thus, instead of using 0.7 to 1.5 ml of buffer with 200 mg of insoluble dye-amylose comple— 1.0 to 5.0 ml can be used, with 4.5 ml preferred. All other reaction conditions, concentrations, amounts and materials are the same for both the dynamic and static system.

In both the dynamic and static systems, the temperature is critical within a narrow range to insure that the stability of the system is not deleteriously effected, thus 37° to 40°C is operable, with 40° preferred. The amount of enzyme solution present is critical only insofar as economics, convenience and suspected enzyme activity is concerned. Thus, under ordinary circumstances, a satisfactory color test can be obtained if 0.1 to 0.4 ml of test solution per 200 mg of substrate is utilized. The length of time the enzyme activity is tested is dictated by economics and convenience. Thus, generally 15 minutes incubation will be sufficient to determine the relative activity and amount of enzyme present. Longer times are uneconomical and shorter times are generally insufficient to produce adequate amounts of solubilized dye to permit consistent, accurate readings.

In order to stop the activity of the enzyme at a predetermined time, it is necessary to quench the system. This can be accomplished in several ways, e.g., change the pH of the system or remove the product. The quench method preferred according to this invention, is to change the pH. This can be accomplished by making it either alkaline, e.g., at least 9, preferably 10 to 11, or acidic, e.g., 3.5 to 4.5, preferably 4.0. The alkaline quench is suitable for systems which contain substantial amounts of protein while the acidic quench is suitable for those systems which contain little or no protein.

The alkaline quench is accomplished by adding an alkaline material which is of sufficient strength to require little volume to raise the pH; preferred are organic materials, e.g., lower alkanol amines such as 2-amino-2-methyl-1-propanol and (trihydroxy methyl)amin methane. "Lower alkanol amines" are defined herein as alkanol amines containing from 2-5 carbon atoms.

The acidic quench is accomplished by adding an acidic material which is of sufficient strength to require little volume to lower the pH but is not so strong that it will deleteriously affect the components of the system. Preferred are organic materials, for example, lower alkyl carboxylic acids, i.e. those containing from 1 to 4 carbon atoms, such as acetic acid.

After the quench, the mixture is diluted to a suitable volume, usually 10 ml, and centrifuged or filtered to remove all the insoluble material. The absorbence of the supernatant or filtrate, which should be transparent and of the same color as the dye, is then compared against a standard or control.

The dye is solubilized when the enzyme attacks the starch portion of the complex. Since the enzyme hydrolyzes the linear starch in a regular rather than random manner, the amount of dye solubilized at any given time by a particular enzyme activity is reproducible. This has been found to be the case in the test of the invention wherein a reproducibility within 2% to 4% of the mean is accomplished.

The materials utilized in the amylase assay can be provided in, for example, a convenient kit comprising the substrate, the diluent and a standard. The substrate can be provided, for example, in a container, e.g., a capsule, and is composed of the dyed amylase substrate, sodium chloride and the buffer.

The diluent can be provided in suitable containers, e.g., packets containing crystals of the buffer having a certain desired pH when dissolved in water, and ready for dissolution in water.

The standard solution can be provided in a suitable container, e.g., a test tube or flask and contains a measured amount of dye solution.

The dye-amylose complex is applicable to amylase assay of both plant and animal origin as illustrated by the examples given below.

Of the following Examples, Nos. 2-4 illustrate the invention, and Example 1 describes the preparation of a water-insoluble complex for use in the invention. All temperatures given in the Examples are in degrees centigrade.

Preparation of dye-starch complex.

a) 10 g of Cibacron (Registered Trade Mark) Brilliant Blue F3GA in 1 liter of water is added to a suspension of 100 g of amylose in 1 liter of water at 50°, with stirring. 200 g of anhydrous sodium sulfate is slowly added over a period of 30 minutes, with stirring, then 150 ml of 10% tri-

sodium phosphate is added. The reaction is allowed to proceed at 50° for 75 minutes, with continuous stirring. The resulting suspension is stirred overnight at room temperature, transferred to a centrifuge and centrifuged 20 minutes at 2500-3000 rpm. The precipitate is recovered, rewashed, recentrifuged, recovered and then dried. The product is a blue powdery material of a complex of amylose and Cibacron (Registered Trade Mark) Brilliant Blue F3GA containing one dye molecule for about every seven to about every ten sugar moieties in the amylose.

b) By substituting equal amounts of Cibacron (Registered Trade Mark) Brilliant Orange G.E. for Cibacron (Registered Trade Mark) Brilliant Blue F3GA and following the procedure of Example 1a, an orange powdery material of a complex of amylose and Cibacron (Registered Trade Mark) Brilliant Orange G.E. containing one dye molecule for about every seven to about every ten sugar moieties in the amylose is obtained.

Example 2

Determination of amylase activity.

Ml. enzyme solution	$A_{625\ m\mu}$	*mg dye released/spec.*	*Mean*	*Mg dye/ml*
0.1	0.22; 0.21	0.137; 0.131	0.134	1.34
0.2	0.43; 0.41	0.256; 0.269	0.262	1.31
0.3	0.60; 0.61	0.375; 0.381	0.378	1.26
0.4	0.80; 0.83	0.500; 0.519	0.509	1.27

b) 0.2 ml enzyme solution containing 50-250 amylase units (as determined reductrimetrically) is added to 4.5 ml of an 0.5-2.5 percent suspension of Brilliant Orange G.E.-amylose complex in 0.02-0.1 M phosphate buffer, pH 7.0, containing 0.01-0.05 mol/l sodium chloride. The mixture is heated 30 minutes at 40°.

At the end of the incubation period, 2 ml of 1N acetic acid are added and the resulting insoluble material is removed. The absorbance of the remaining solution is measured against a control in a spectrophotometer at 490 mμ.

The control is prepared as described above except distilled water is substituted for the enzyme sample.

Results: The results are shown as the amount of dye released per amount of reducing sugar formed (expressed as glucose).

Enzyme Units (Glucose/100 ml)	*Dye Units* ($A_{490} \times 10^3$)	*Dye/Glucose*
50	80	1.6
125	345	2.76
250	680	2.72

Example 3

Preparation of Reagent Test Kit.

A reagent test kit containing sufficient material for 100 tests and comprising the following is utilized:

a) substrate

a) In a 25 ml Erlenmeyer flask, 200 mg finely ground Cibacron (Registered Trade Mark) Brilliant Blue F3GA-amylose is suspended in 4.5 ml 0.04 M phosphate buffer, pH 7.0, containing 0.05 mol/l sodium chloride and warmed to 40°. The enzyme solution, 0.1-0.4 ml, is added and the mixture is incubated at 40° for 15 minutes in a Dubnoff shaker. One ml of 2-amino-2-methyl-1-propanol (0.5 M, pH 10.25) is added and the entire mixture is diluted to 10.0 ml and centrifuged. The absorbance of the supernatant is obtained at 625 mμ against a reagent blank.

The reagent blank is prepared as described above except 0.1 ml-0.4 ml of water is substituted for the enzyme sample.

A calibration curve is prepared by measuring the absorbance of solutions of Cibacron (Registered Trade Mark) Brilliant Blue F3GA containing 100 to 400 μg dye in 10 ml mixed phosphate-alkanolamine buffer.

Results: Hog pancreatic α-amylase diluted 1:10,000, served as the enzyme source and the activity is expressed as mg dye formed by 1.0 ml dilute enzyme.

200 capsules, each containing the following ingredients expressed as milligrams per capsule:

amylase substrate	200
sodium phosphate monobasic	1.94
sodium phosphate dibasic anhydrous	2.99
sodium chloride	0.93

b) diluent

1.38 g of crystals of $NaH_2PO_4.H_2O$; pH 4.2 when dissolved in water.

c) stock standard

15 ml of a standard containing 29.2 mg/100 ml prepared as follows:

About 292.0 mg of Cibacron (Registered Trade Mark) Brilliant Blue F3GA is dissolved in 1 liter of distilled water and then a 15 ml aliquot is put into a capped glass container.

Example 4

Determination of amylase activity.

The contents of 1 substrate capsule (as prepared in Example 3) and 0.8 ml of distilled water are added to each of two test tubes marked "Test" and "Blank". The resultant slurry is mixed thoroughly. Both tubes are incubated for 10 minutes at 37°.

0.1 ml of serum is added to the tube marked "Test" and mixed vigorously. Both tubes are then incubated for exactly

15 minutes at 37°.

10 ml of the diluent, prepared by dissolving 1.38 g of $NaH_2PO_4.H_2O$ in deionized or distilled water, diluted to 100 ml, is added to both tubes. 0.1 ml serum is added to the "Blank". The contents of the tubes are mixed well and centrifuged to obtain a clear supernatant.

The optical density of the "Test" solution at 625 mμ (red filter) is then compared to the "Blank" and the amylase activity is determined from a standard curve.

The following indicates the results expressed as the amount of dye released per 0.1 ml of test solution using different serum specimens:

Specimen No.	*mg dye/0.1 ml*
1	0.085
2	0.074
3	0.077
4	0.110
5	0.103
6	0.069
7	0.118
8	0.072
9	0.118
Pooled Serum Control	0.072
Mean	0.089

WHAT WE CLAIM IS:—

1. A method for determining the α-amylase activity of a test material, wherein a water-insoluble complex composed of a water-soluble reactive dye as hereinbefore defined and an amylose containing material is subjected for a suitable time, to the enzymatic action of the test material in an aqueous phosphate buffer at a pH of from 6·9 to 7·1 and at a temperature of from 37°C to 40°C and containing at least 0.01 mol/l of an alkali metal chloride catalyst, the reaction is quenched and the colour of the supernatant liquid is compared with a standard.

2. A method as claimed in claim 1, wherein a complex is used the dye component of which is a monochlorotriazine with an anthraquinone disulfonate substituent.

3. A method as claimed in claim 1 or 2, wherein the alkali metal chloride catalyst is sodium chloride.

4. A method as claimed in any one of claims 1 to 3 wherein the test reaction is quenched by raising the pH of the test meduim to at least 9 after 15 minutes, with a lower alkanol amine.

5. A method as claimed in claim 4, wherein the lower alkanol amine is 2-amino-2-methyl-1-propanol.

6. A method as claimed in any one of claims 1 to 3, wherein the test reaction, is quenched by lowering the pH of the test medium with acetic acid.

7. A method for determining the α-amylase activity of a test material, substantially as hereinbefore described with reference to the foregoing Examples 2 and 4.

8. A reagent test kit for determining amylase activity, comprised of separate containers of (a) buffered (pH 6·9 to 7·1) solution of a water-insoluble complex, composed of a water-soluble reactive dye as hereinbefore defined and an amylose containing material, and sodium chloride, (b) a crystalline phosphate having a pH of 4·2 when dissolved in water, and (c) as a standard, a solution of water soluble reactive dye as hereinbefore defined.

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